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A BAFF antagonist suppresses experimental autoimmune encephalomyelitis by targeting cell-mediated and humoral immune responses

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Abstract

BAFF [B cell-activating factor of the tumour necrosis factor (TNF) family] and APRIL (a proliferation-inducing ligand) are two TNF family members with shared receptors. While, physiological roles for APRIL are not fully understood, BAFF is critical for B cell homeostasis and also acts as a co-stimulator of T cells. Using a B and T cell-mediated mouse model of multiple sclerosis (MS), myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE), we observed that a BAFF/APRIL antagonist (soluble BCMA-Fc) inhibited central nervous system inflammation and demyelination such that it suppressed the onset and progression of clinical symptoms of EAE. In addition to dramatically reducing the titre of MOG-specific auto-antibodies, this treatment also induced a switch in the subtype of the T_h cell population characterized by marked alterations in cytokine production following re-stimulation with MOG *in vitro*. Indeed, hBCMA-Fc therapy led to significant increases in the level of transforming growth factor β, while the levels of T_h1 cytokines were markedly diminished. These results not only identify BAFF as a critical factor in maintaining humoral immunity in EAE but also support its role in T lymphocyte responses. Our findings demonstrate that hBCMA-Fc acts on both effector arms of the immune response in EAE, a characteristic that may be of significant therapeutic value in the treatment of MS.

Introduction

Experimental autoimmune encephalomyelitis (EAE), a chronic inflammatory demyelinating disease of the central nervous system (CNS), is currently viewed as the most accurate animal model of the human autoimmune disease, multiple sclerosis (MS) (1). EAE can be induced via immunization with self-antigens derived from CNS myelin, such as MBP, proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG), or by adoptive transfer of $CD4^+$ T cells reactive to such antigens (1).

MOG-induced EAE (herein referred to as MOG-EAE) is characterized by perivascular inflammation and extensive primary plaque-like demyelination in the CNS of affected animals. The main clinical symptoms of MOG-EAE are paraparesis and paralysis but optic neuritis, a common symptom in MS, is also a characteristic that has not been reported in other animal models (2, 3). The humoral and cellular arms of the immune system synergise to elicit the pathological features of this disease, as high anti-MOG antibody titres and MOG-specific autoreactive T cells are found in affected animals (4). Supporting the use of this model is the observation that T cells isolated from the peripheral blood (PB) of MS patients show a greater proliferative response to MOG compared with MBP,

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PLP or myelin-associated glycoprotein (MAG) (5). Evidence also suggests that conformation-dependent anti-MOG antibodies play a key role in demyelination in non-human primate models of MS (6–10) and in MS itself (7). Furthermore, MOG-reactive antibodies in the sera of patients presenting with an initial demyelinating event are predictive of clinically definite MS (11). We have recently reported the crystal structure of the MOG extracellular domain and have shown that MOG forms a head to tail homophylic dimer (12). The production of conformation-dependent antibodies generated in the immune response to native (MS) or refolded recombinant myelin oligodendrocyte glycoprotein (rMOG) (EAE) (10) may elicit their effects by disrupting the homodimer interface observed in this structure (12). This is supported by the observation that the conformation-dependent, demyelinating antibody 818C5 (13) binds to a region on the MOG extracellular domain which is likely to cause disruption of this homodimer interface (14). Antibody-mediated cross-linking of MOG and/or disruption of the dimer may elicit pathological events including changes in myelin morphology as has been observed for oligodendrocytes *in vitro* (15) as well as the unveiling of buried epitopes at the dimer interface (8).

Traditionally, MS and EAE have been viewed as $CD4^+$ T cell-mediated conditions; however, evidence also indicates that the humoral immune response may also be important in the induction stages of these diseases. The ability of MOG peptide residues 35–55 (MOG_{35-55}) and the inability of rMOG to induce disease in B cell-deficient mice indicate that B lymphocytes are critical for an aspect of the initiation of MOG-EAE (16). This observation has since been attributed to the lack of MOG-specific antibody, as the transfer of rMOG-primed serum led to the induction of disease in rMOG-primed B cell-deficient mice (17).

The pathological role of MOG-specific antibodies in MS suggests that targeting the humoral immune response may be an avenue for therapy. Specific antagonists to BAFF [B cell-activating factor of the tumour necrosis factor (TNF) family] (18), a key cytokine for normal B cell development (19), are likely candidates for such intervention (20, 21). Ligands from the TNF superfamily are essential in mediating an effective immune response in mammals (22). Unregulated activity of some of these ligands has also been shown to participate pathologically in autoimmune disorders by either inducing apoptosis or promoting aberrant cell survival, proliferation and/or maturation (22, 23). BAFF, a member of the latter group, is expressed by T cells (24), monocytes, dendritic cells and neutrophils as either a membrane bound or secreted form and has been well documented as a crucial factor in B cell survival and maturation (19). Over-expression of a BAFF transgene in mice results in an accumulation of B cells expressing high levels of MHC class II and Bcl-2 (25). In addition, these mice possess elevated Ig levels and spontaneously secrete autoantibodies, including antibodies to DNA which eventually lead to an autoimmune manifestation similar to systemic lupus erythematosus (SLE). Three receptors for BAFF have been identified so far: transmembrane activator and CAML-interactor (TACI) (26, 27), B cell maturation antigen (BCMA) (26–28) and BAFF-R (29, 30). While BCMA and TACI may also bind a proliferation-inducing ligand (APRIL) (27, 31–33), BAFF-R only binds BAFF and appears to be chiefly responsible for the physiological effects of BAFF on B cells (29). Macrophages and epithelial cells secrete APRIL, which has been described as a tumour-stimulating factor with high levels being recorded in many types of cancers. The generation of APRIL knockout mice has demonstrated that APRIL is not required for B cell survival/development (34, 35) and experiments using BAFF-R- and BAFF-deficient mice suggest that APRIL cannot compensate for the loss of BAFF (29).

It now appears that BAFF has important roles in T cell function. Upon TCR ligation, BAFF provides a potent co-stimulatory signal to human T cells enhancing their division and IL-2 production (36). BAFF production by antigen-presenting cells (APC) can be up-regulated following exposure to bacteria while blockade of endogenous BAFF inhibits T cell activation (24). This suggests that BAFF may have a critical role in T cell–APC interactions. Furthermore, hTACI-Fc is able to impair activation of T cells when re-stimulated *in vitro* and T cell priming *in vivo* (37). The diminished T cell activation observed correlates with reduced inflammation and bone/cartilage destruction in a mouse model of rheumatoid arthritis. The recent discovery that BAFF-R is expressed on activated and memory T cells, while BCMA and TACI are not, indicates that BAFF co-stimulation of T cells is mediated through the BAFF-R (38). These data are further supported by the finding that T cells from A/WySnJ fail to receive BAFF co-stimulation following CD3 activation (38).

Since BAFF is such a strong promoter of B cell survival and maturation as well as a co-stimulator of T cells, we have investigated the efficacy of preventing BAFF signalling as a means of suppressing B cell antigen presentation and antibody production, as well as T cell co-stimulation, in the induction and effector phases of EAE. This was achieved by administration of soluble human BCMA fused to the constant region of human IgG1 (hBCMA-Fc). We observed that the onset and severity of MOG-EAE were significantly retarded in mice pre-treated with hBCMA-Fc. Mice treated with hBCMA-Fc after disease onset showed a significantly improved clinical course that correlated with histopathological analysis. Both treatments corresponded to a reduction in B cell number and MOG-specific antibody titre in sera as well as a T_h2/T_h3 T cell response [high transforming growth factor (TGF)-β levels and a decrease in IFN-γ]. Our findings suggest that sequestration of BAFF may be an effective means of treating autoimmune manifestations involving aberrant B and T cell responses, such as MS.

Methods

Induction and treatment of EAE

Female 8- to 12-week old non-obese diabetic (NOD)/Lt and C57BL/6 mice (Animal Resource Center, Perth, Australia and Central Animal House, La Trobe University, Melbourne, Australia) were immunized subcutaneously with 100 μg of human rMOG extracellular domain residues 1–121 (rMOG) emulsified in CFA (Difco, BD, North Ryde, Australia) supplemented with *Mycobacterium tuberculosis* (4 mg ml^{-1}) (2). Mice immediately received 300 ng of pertussis vaccine (List Biological Laboratories, Campbell, CA, USA) intravenously and again 48 h later. Mice were monitored daily for clinical

signs of neurological impairment and graded as follows: 0 = no detectable impairment, 1 = limp tail and loss of weight, 2 = weakness of hindlimbs, 3 = complete paralysis of one/both hindlimbs, 4 = complete paralysis of one/both hindlimbs and ascending paralysis; at this stage, mice are considered moribund and are euthanized. The individual scores from the day of treatment onset were recorded, with the mean cumulative change in clinical score from each group on each day being determined. Mice were treated intra-peritoneally every second day with either 100 μg of recombinant hBCMA-Fc (28), 100 μg of polyclonal hIg (Novartis, Basel, Switzerland) or PBS upon reaching a clinical score of 2. In pre-treatment trials, administration of 50 μg of hBCMA-Fc, 50 μg polyclonal IgG or PBS commenced 5 days prior to immunization. All experiments involving animals and their care were performed using an approved protocol of the La Trobe University Animal Ethics Committee, in accordance with the Australian code of practice for the care and use of animals for scientific purposes [National Health and Medical Research Council (Australia), 1997].

Measurement of MOG-specific antibodies

Serum anti-MOG titre and relative isotype concentrations were determined by ELISA. Briefly, microtitre plates were coated with rMOG (5 μg ml^{-1}), blocked with 3% BSA (Sigma, St Louis, MO, USA), washed and incubated with 1:3 serial dilutions of test sera. Total Ig and isotypes bound to MOG were detected using HRP-coupled anti-Ig, -IgG1, -IgG2b, -IgG3 and -IgM (Southern Biotechnology Associates Inc., Birmingham, AL, USA) developed with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (450 ng ml^{-1}) and the optical density measured at 405 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Relative IgG2c (39) levels were determined in the same way except biotinylated-IgG2c (anti-mouse $IgG2_a^c$, BD Biosciences PharMingen, San Diego, CA, USA) and HRP–streptavidin (Pierce Biotechnology, Rockford, IL, USA) were used as the primary detection reagents.

Antigen-specific T cell responses

Spleens (SPs) and lymph nodes (LNs) were taken from mice sacrificed at days 56 and 51 post-immunization for NOD/Lt and C57BL/6 mice, respectively. Single-cell suspensions were prepared and cultured in microtitre plates at 2×10^5cells per well in RPMI 1640 (Invitrogen, Paisley, Scotland) containing 10% heat-inactivated FCS (CSL, Melbourne, Australia), 2×10^{-5} M (2-mercaptoethanol, 2 mM L-glutamine, 1 mM Na pyruvate, 10 mM HEPES, 100 U ml^{-1} of penicillin and 100 μg ml^{-1} of streptomycin (Invitrogen)). Cells were cultured in the presence of various stimuli: anti-CD3 (10 μg ml^{-1}; Pharmingen), rMOG (20 μg ml^{-1}), MOG_{35-55} peptide (20 μg ml^{-1}; M-E-V-G-W-Y-R-S-P-F-S-R-V-V-H-L-Y-R-N-G-K; Auspep, Melbourne, Australia), recombinant bovine butyrophilin (20 μg ml^{-1}), Concanavalin A (5 μg ml^{-1}; Sigma) or culture medium alone. Proliferation was measured by addition of [^{3}H]thymidine ([^{3}H]TdR, 1 μCi per well; Amersham Biosciences, Buckinghamshire, UK) for the last 16 h of an 88-h culture; [^{3}H]TdR incorporation was determined by liquid scintillation counting (Wallac/PerkinElmer, Boston, MA, USA). To investigate the role of hBCMA-Fc on splenocytes *in vitro*, 10 μg of hBCMA-Fc was added to splenocyte cultures from pre-treated mice using the conditions described above. Ten micrograms of hIg and culture media alone were used as controls. Cytokine profiles were determined from supernatants of splenocyte cultures following a 48-h incubation with culture medium alone or in the presence of rMOG (20 μg ml^{-1}). The levels of IL-2, IL-4, IL-6, granulocyte macrophage colony-stimulating factor (GM-CSF), TGF-β and IFN-γ secreted by splenocytes were determined via a sandwich ELISA using matched mAb pairs and performed according to the manufacturer's instructions (BD Biosciences PharMingen). Recombinant mouse standards (Peprotech, Rocky Hills, NJ, USA) were used to generate standard curves.

Flow cytometry

Single-lymphocyte suspensions from SP, LNs and PB were prepared. Whole blood was treated with 25 IU ml^{-1} of heparin (Sigma), plasma was removed and erythrocytes lysed in 5 ml of ACK lysis buffer (0.15 M NH_4Cl, 10.0 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.3) for 3 min at room temperature, then washed and re-suspended in PBS. Cell populations were identified by fluorochrome-conjugated mAbs to CD5, CD4, CD3, CD8, CD19, CD45R and CD138 (PharMingen). Cell-associated fluorescence was analysed on a FACSCalibur flow cytometer using the Cellquest software (BD, North Ryde, Australia).

Histological examination

Upon CO_2 asphyxiation, brain and spinal cord were immersion fixed in 4% formalin and embedded in paraffin blocks. Blocks were cut in the caudal to rostral direction and sequential sections stained with Luxol fast blue (LFB) or hematoxylin–eosin (H&E). Stained sections were examined for the level of demyelination and perivascular infiltration using a conventional microscope while blind to the treatment regimen.

Statistical analysis

Experimental data were statistically analysed using one-way analysis of variance tests with either Tukey HSD or Scheffe post hoc analysis and Student's *t*-test.

Results

Pre-treatment with hBCMA-Fc delays the onset and severity of MOG-EAE

Given the importance of BAFF in humoral immunity and the finding that BAFF is involved in T cell co-stimulation (24, 36–38), we hypothesized that treatment with hBCMA-Fc prior to immunization with the encephalitogen may block the development of EAE. To test this, C57BL/6 mice were administered soluble hBCMA-Fc fusion protein every second day, beginning 5 days prior to immunization with rMOG/CFA (day 5) and finishing on experimental completion (day 30). As highlighted in Table 1, hBCMA-Fc pre-treatment significantly delayed the onset of disease by 5 and 7 days compared with control hIg and PBS pre-treatments, respectively. Furthermore, the incidence of EAE in mice pre-treated with hBCMA-Fc was 20% lower than with hIg and 60% lower than with PBS. When EAE did develop, its progression was much slower in mice pre-treated with hBCMA-Fc with the mean clinical score

of 1 being reached 11 and 13 days later than hIg and PBS pre-treatment, respectively, resulting in the clinical score of mice treated with hBCMA-Fc being significantly lower than hIg on day 16 ($P = 0.0399$), day 17 ($P = 0.0341$), day 19 ($P = 0.0395$) and day 20 ($P = 0.0468$; Table 1 and Fig. 1).

Administration of hBCMA-Fc significantly decreased the number of $CD19^+$ cells in the tissues analysed ($P < 0.0001$), with no difference observed in the numbers or ratio between $CD4^+CD3^+$ and $CD8^+CD3^+$ T cells (Fig. 2A and B). Of the B lymphocytes remaining after hBCMA-Fc treatment, plasma cell ($CD138^+$) and B1 B cell ($CD5_{low}B220_{low}$) populations were relatively normal, except for a significant decrease in splenic plasma cells ($P = 0.0182$, hIg; $P = 0.0335$, PBS; Fig. 2B). Mice receiving hBCMA-Fc had a significantly impaired humoral immune response with the titre of rMOG-specific serum antibodies reduced >3-fold compared with controls ($P = 0.0004$, hIg; $P = 0.015$, PBS; Fig. 2C). Finally, the serum levels of rMOG-specific Ig isotypes following hBCMA-Fc pre-treatment were all reduced compared with controls, including a significant reduction in IgG2c (39) ($P = 0.006$, hIg; $P = 0.002$, PBS; Fig. 2C).

Prophylactic treatment of C57BL/6 mice with hBCMA-Fc suppressed the activation of T cells compared with those from control-treated mice when re-stimulated *in vitro* with rMOG ($P = 0.03$, hIg; $P = 0.01$, PBS) or anti-CD3 mAb (Fig. 3A). In addition, splenocytes from hBCMA-Fc pre-treated mice secreted lower levels of T_h1 cytokines, including a significant decrease in IFN-γ ($P < 0.009$), as well as a significant increase in the T_h2/T_h3 cytokine TGF-β ($P < 0.05$), when re-stimulated with rMOG *in vitro* (Fig. 3B).

Since we have demonstrated that splenic T cell activation is inhibited in cells isolated from hBCMA-Fc-treated mice, we were interested to determine whether hBCMA-Fc could exert a similar effect directly on MOG-sensitized splenocytes isolated from the control mice. To test this, hBCMA-Fc or hIg, in the presence of rMOG or anti-CD3 mAb, was added to cultures of splenocytes taken from PBS-treated animals. Splenocytes had a significantly decreased proliferative response to rMOG ($P = 0.007$, hIg; $P = 0.02$, PBS) in the presence of hBCMA-Fc *in vitro* (Fig. 3C).

Table 1. Clinical response in C57BL/6 mice treated 5 days prior to immunization

Treatment	Incidence of EAE (%)	Onset of EAE[a]	Day mean clinical score reached 1	Number of mortalities to EAE
hBCMA-Fc	40	19.5 ± 2.5	Day 27	0
hIg	60	14.5 ± 1.1	Day 16	0
PBS	100	12.5 ± 0.69	Day 14	2

[a]Mean day that clinical symptoms were first observed in sick mice ± SEM.

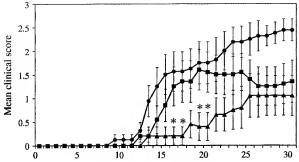

Fig. 1. hBCMA-Fc delays the induction and reduces the frequency of MOG-EAE. C57BL/6 mice were pre-treated daily with intra-peritoneal injections of 50 μg of hBCMA-Fc (filled triangles), control hIg (filled squares) or PBS (filled circles) commencing 5 days prior to immunization with rMOG in CFA ($n = 10$, 10 and 6, respectively). Mice were scored daily in a blind manner and treated every alternate day post-immunization. Data represent mean scores ± SEM. Clinical score of mice treated with hBCMA-Fc is significantly lower than hIg on day 16 ($P = 0.0399$), day 17 ($P = 0.0341$), day 19 ($P = 0.0395$) and day 20 ($P = 0.0468$). Onset of EAE is significantly delayed by hBCMA-Fc pre-treatment ($P < 0.05$).

hBCMA-Fc prevents progression and aids recovery from established EAE

Since pre-treatment with hBCMA-Fc was able to delay the induction of MOG-EAE by suppressing pathogenic B and T cell responses, we next evaluated whether this treatment could improve clinical symptoms of established MOG-EAE by inhibiting such events. To investigate the effect of BCMA-Fc on MOG-EAE, NOD/Lt and C57BL/6 mice were immunized with rhMOG in CFA, monitored daily and separated into treatment groups upon displaying clinical symptoms. Mice were either treated with human BCMA-Fc, polyclonal human Ig or PBS and monitored closely for 7–8 weeks. A chronic-relapsing disease pattern was present in our MOG-EAE model, with clinical symptoms becoming apparent between 12 and 18 days post-immunization. Following the onset of treatment, NOD/Lt mice in the two control groups continued to develop clinical symptoms typical of EAE, whereas mice receiving hBCMA-Fc displayed clear signs of recovery, commencing 9 days after the start of treatment (Fig. 4A). As highlighted in Table 2, BCMA-Fc treatment was able to halt the progression of EAE with only two out of seven NOD/Lt mice showing a net worsening of symptoms. In contrast, control mice showed marked deterioration in health (hIg—6 out of 9; PBS—7 out of 8). Furthermore, treatment with hBCMA-Fc alleviated symptoms, decreasing the clinical score (−0.5 ± 0.39) over the treatment period, with two mice making a complete recovery. In the hIg (+0.7 ± 0.22) and PBS (+0.8 ± 0.19) treatment groups, a normal progression of symptoms was observed, notably resulting in six deaths from EAE in the PBS treatment group. C57BL/6 mice saw a similar but less dramatic difference between treatment groups (Fig. 4B). Table 2 illustrates the beneficial effects of hBCMA-Fc treatment, made obvious by a decrease in clinical score (−0.3 ± 0.18). In comparison, control groups displayed clinical worsening after treatment (hIg = +0.2 ± 0.11 and PBS = +0.2 ± 0.19). In summary, our findings demonstrate that treatment with the decoy receptor, hBCMA-Fc, blocks the progression and decreases the symptoms of MOG-EAE. NOD/Lt mice, treated by intra-peritoneal administration of hBCMA-Fc, showed a significant overall improvement ($F(2,21) = 4.76$; $P < 0.05$), with a net decrease in clinical score equivalent to 1.2 and 1.3 compared with hIg and

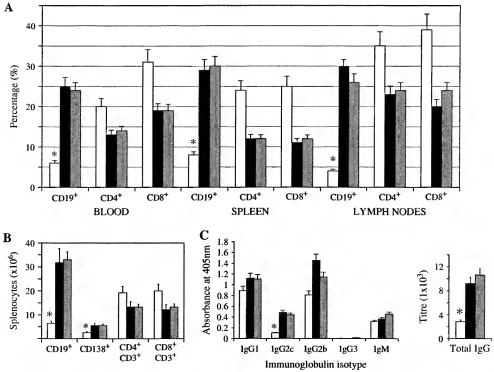

Fig. 2. Treatment with hBCMA-Fc causes B cell loss and inhibits antigen-specific and total Ig production. C57BL/6 mice treated before and after immunization with hBCMA-Fc (white bars), hIg (black bars) and PBS (grey bars) were sacrificed on day 30 and blood and secondary lymphoid organs analysed for lymphocyte composition ($n = 6$ for all groups). (A) The percentage of B cells (CD19+, *$P < 0.0001$), T_h cells (CD4+CD3+, $P < 0.0001$) and CTLs (CD8+CD3+, $P < 0.0001$) was determined by FACS staining. (B) hBCMA-Fc significantly reduced the number of splenocytes ($P < 0.01$; data not shown), resulting in fewer splenic B cells (*$P < 0.0001$) and plasma cells (*$P = 0.0182$, hIg; $P = 0.0335$, PBS) while not affecting T cell numbers ($P = 0.061$). (C) Relative levels of serum anti-rMOG isotypes (IgG2c; *$P = 0.006$, hIg; *$P = 0.002$, PBS) and titre of total anti-rMOG IgG (*$P = 0.0004$, hIg; $P = 0.015$, PBS) on day 30 following treatment were determined by ELISA. Results represent mean comparable binding of each group ± SEM. hBCMA-Fc, $n = 10$; hIg, $n = 10$ and PBS, $n = 6$.

PBS, respectively (Table 2). A similar but less-pronounced effect was observed in the hBCMA-Fc-treated C57BL/6 mice, with a decrease in clinical score equivalent to 0.5 over both control groups (Fig. 4B, Table 2).

Humoral responses are severely impaired as a direct result of hBCMA-Fc treatment

Considering the importance of autoreactive antibodies in EAE and MS, as well as the pivotal role of BAFF in B cell maintenance, we next investigated the effect of hBCMA-Fc on the humoral immune response. In both mouse strains used, hBCMA-Fc significantly decreased the B cell population in PB, LNs and SP, with mice receiving hBCMA-Fc possessing 4–5 times fewer conventional B cells ($CD19^+$) compared with control mice ($P < 0.0001$; Fig. 5A and B). It is also important to note that the total number of lymphocytes in the secondary lymphoid organs of hBCMA-Fc-treated mice was notably reduced (data not shown). The rMOG-specific antibody titre was decreased ~5-fold in both strains ($P < 0.05$) following hBCMA-Fc treatment, with the NOD/Lt mice (1/13 333) having a lower titre than the C57BL/6 mice (1/23 000) (Fig. 5C and D). In addition, the isotype population of rMOG-specific antibody present in NOD/Lt mice receiving hBCMA-Fc treatment was deficient in IgG3, while IgG1, IgG2b and IgG2c were substantially reduced when compared with mice receiving the control treatments (Fig. 5C). In contrast, IgG2b and IgM were increased in C57BL/6 receiving hBCMA-Fc (Fig. 5D). The level of serum neutralizing anti-hBCMA-Fc antibody was determined, as a high concentration can dampen the effect of treatment. Only low levels of antibody to hBCMA-Fc were detected in the sera of both strains, at a concentration of 1.4 $\mu g\ ml^{-1}$ (NOD/Lt) and 1.3 $\mu g\ ml^{-1}$ (C57BL/6) (data not shown). Thus, these findings support the notion of MOG-specific antibodies participating in the progression of MOG-EAE, and suggest one mechanism by which hBCMA-Fc ameliorates the severity of MOG-EAE by dramatically reducing the production of such antibodies.

hBCMA-Fc treatment impairs T cell activation in vivo, and initiates a switch from a T_h1 to a T_h2/T_h3 response in the SP

We next investigated the role of BAFF-mediated activation of T cells *in vivo* and *in vitro* from mice with established MOG-EAE. Mice treated with hBCMA-Fc have a normal proportion of T_h cells ($CD4^+CD3^+$) and CTL ($CD8^+CD3^+$) cells with no significant difference in the ratio of the $CD4^+$:$CD8^+$ being observed between groups (Fig. 5A and B). To determine the effect of hBCMA-Fc on T cell responses *in vivo*, SPs and draining lymph nodes (DLNs) of rMOG-immunized mice were

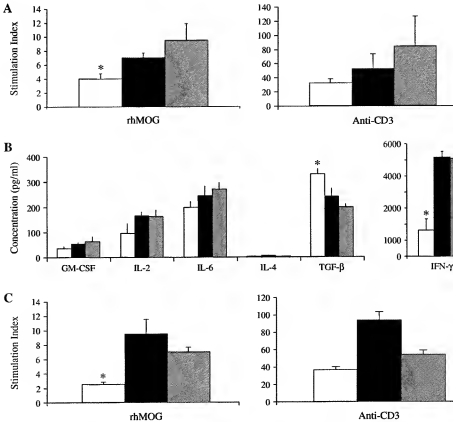

Fig. 3. hBCMA-Fc pre-treatment inhibits full T cell responses *in vivo* and *in vitro*. (A) Splenocytes from C57BL/6 mice treated with hBCMA-Fc (white bars), hIg (black bars) and PBS (grey bars) were harvested at day 30 and re-stimulated *in vitro* with rMOG and anti-CD3 mAb for 72 h. hBCMA-Fc treatment *in vivo* resulted in significantly less T cell proliferation to rMOG *in vitro* (**P* = 0.03, hIg; *P* = 0.01, PBS). Bars represent the mean stimulation index [SI; mean counts per minute (c.p.m.) in wells containing rhMOG or anti-CD3 mAb divided by the mean c.p.m. in wells containing RPMI only] ± SEM. Mean c.p.m. with media alone were 1411 (hBCMA-Fc, *n* = 10), 658 (hIg, *n* = 10) and 887 (PBS, *n* = 6) c.p.m. (B) Splenocytes were cultured in the presence of rMOG for 48 h, the supernatants were then removed and cytokine concentrations determined by ELISA. IFN-γ levels were significantly different from hIg control (**P* < 0.009), as was TGF-β (**P* < 0.05). hBCMA-Fc, *n* = 10; hIg, *n* = 10 and PBS, *n* = 8. (C) We next investigated whether hBCMA-Fc could inhibit T cell stimulation *in vitro* by adding 10 μg ml^{-1} of either hBCMA-Fc (white bars), hIg (black bars) or RPMI alone (grey bars) into a culture of splenocytes from a PBS-treated mouse in the presence of rMOG (**P* = 0.007, hIg; *P* = 0.02, PBS) or anti-CD3 mAb. Bars represent the mean SI ± SEM of triplicate wells in one experiment. hBCMA-Fc, *n* = 8; hIg, *n* = 9 and PBS, *n* = 6.

removed. Both the SPs and the DLNs from hBCMA-Fc-treated mice were discernibly smaller than those from control-treated mice (data not shown). When re-stimulated *in vitro* with rMOG or anti-CD3 mAb, splenocytes from hBCMA-Fc-treated mice of both strains had a reduced proliferative response as determined by [^{3}H]TdR incorporation (Fig. 6A and B). Compared with control mice, splenocytes from hBCMA-Fc-treated NOD/Lt mice produced significantly less IFN-γ ($P < 0.05$) along with decreased levels of IL-2, IL-6 and GM-CSF, while secreting significantly more TGF-β ($P < 0.008$) in response to rMOG (Fig. 6C). In contrast, no obvious difference in cytokine production existed between the splenocytes of C57BL/6 mice receiving hBCMA-Fc and control treatments, except for hIg-treated mice, which had an increased concentration of most cytokines tested (Fig. 6D). Collectively, these results indicate that hBCMA-Fc inhibits the proliferative capacity of splenic T cells, and that this is likely to be an additional mechanism by which hBCMA-Fc treatment blocks the progression of MOG-EAE. Furthermore, the significant increase in the production of TGF-β and the suppression of T_h1 cytokines, particularly IFN-γ, observed in splenocytes of hBCMA-Fc-treated NOD/Lt mice and not C57BL/6 mice could be a possible factor behind the differing responsiveness of each strain to hBCMA-Fc.

hBCMA-Fc reduces inflammation and inhibits lesion formation in the CNS

Given the ability of hBCMA-Fc to significantly decrease the antibody response and the activation of T cells in MOG-EAE, we next examined the resulting effect on CNS pathology. Sections of brain and spinal cord from mice, treated with hBCMA-Fc, hIg or PBS after the onset of EAE, were examined in a blind manner for cellular infiltration and demyelination by H&E and LFB staining, respectively. Histological examination of the CNS tissue was performed on mice sacrificed 55 days (NOD/Lt) and 51 days (C57BL/6) post-onset of treatment. NOD/Lt mice administered with hBCMA-Fc displayed far less inflammatory foci in the spinal cord and cerebellum, with no

obvious signs of inflammatory cell migration in the pons, medulla and forebrain compared with mice receiving control treatments with an equivalent clinical score (Figs 7 and 8). While hBCMA-Fc-treated NOD/Lt mice did possess multiple plaque-like areas of demyelination, these were typically older with very disperse mononuclear and polymorphonuclear cell infiltration and were concentrated in the spinal cord (data not shown) and cerebellum (Fig. 7E and F). The CNS tissue from C57BL/6 mice treated with hBCMA-Fc presented a similar reduction in inflammation in the forebrain, pons, medulla and cerebellum compared with the identical section of clinically matched mice receiving control treatment (data not shown). The C57BL/6 mice also presented a similar level of demyelinating plaque-like lesions; however, these appeared to be generally older with little infiltration in the hBCMA-Fc-treated mice (data not shown).

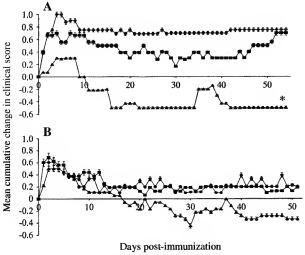

Fig. 4. hBCMA-Fc ameliorates clinical symptoms in MOG-EAE. Mice were immunized with rMOG in CFA and treated with 100 μg of hBCMA-Fc (filled triangles), control hIg (filled squares) or PBS (filled circles) every second day upon reaching a clinical score of 2 (see Methods). (A) NOD/Lt mice displayed a significant recovery following hBCMA-Fc treatment >8 weeks (*$F(2,21) = 4.76$, $P < 0.05$). hBCMA-Fc, $n = 7$; hIg, $n = 9$ and PBS, $n = 8$. (B) The C57BL/6 disease model failed to show significant difference in response to the various treatments. The effect of treatment is shown as the mean cumulative change in clinical score over the treatment period, with a decrease in clinical score (negative change) corresponding to a recovery from symptoms and an increase in clinical score (positive change) corresponding to deterioration in health. hBCMA-Fc, $n = 9$; hIg, $n = 9$ and PBS, $n = 8$.

Discussion

The heterogeneous pathology of autoimmune diseases, such as MS, possesses a significant challenge in the development of effective immune intervention. Current immunomodulation strategies approved for MS are limited and thus there is a need for developing new and more effective therapies. The MOG-EAE model of MS displays a similar spectrum of symptoms characteristic of the human disease (40). It is widely acknowledged that $CD4^+$ T cells are important in the pathogenesis of EAE, as is a critical role of B cells and myelin-specific antibodies, particularly in lesion formation (4, 7).

BAFF is a potent survival factor for B lymphocytes (18, 41), as well as a co-stimulator of T cell activation (24, 36–38). Consequently, mice transgenic for BAFF display dramatic increases in the number of mature B cells and a modest increase in T effector/memory cells, and eventually develop autoimmune conditions such as high levels of Ig-complexes, anti-DNA antibodies and nephritis (25). Interestingly, elevated levels of serum BAFF have been observed in numerous autoimmune syndromes in humans and mice, such as Sjogren's syndrome (42) and SLE (26). BCMA, TACI and BAFF-R all bind BAFF with high affinity, and recombinant FcR fusion derivatives of these receptors have been successfully used *in vivo* to reduce the number of peripheral B cells and inhibit humoral immune responses in various animal models of autoimmune disease such as SLE (NZBWF1 mice) (26) and rheumatoid arthritis [collagen (CL)-induced arthritis] (37). Such treatment would also ultimately reduce the level of $CD4^+$ T cell co-stimulation, thus hindering their full activation. Since $CD4^+$ T cells are important in mediating an inflammatory immune response in EAE, the reduced activation alone may be responsible for the inhibition we have reported here.

In mice pre-treated with hBCMA-Fc, we also observed a dramatic reduction in the number of B cells and Ig titre, which corresponded to a delayed onset and reduced frequency of EAE. Our observation is consistent with the finding that B cell-deficient mice are resistant to EAE induction with rMOG, a phenomenon attributed to the absence of antibody (16, 17, 43). It has also been shown that B cells are potent APCs that are preferentially used in the priming of naive $CD4^+$ T cells to protein antigens (44, 45). Therefore, any reduction in splenic B cell numbers at the time of immunization may result in a reduced efficiency of MOG presentation to T cells. However, since there is a normal T cell response against rMOG in B cell-deficient mice, it appears unlikely that B cells are important for T cell priming in this model (16, 17). Thus, the production of MOG-reactive antibodies appears to be the critical B cell

Table 2. Clinical response to treatment upon development of EAE

	Treatment	Mean clinical score at treatment		Mean change in clinical score	Number of mice whose clinical score			
		Start	End		Decreased	Increased	Unchanged	Deceased
NOD/Lt	hBCMA-Fc	2.6 ± 0.2	2.1 ± 0.6	−0.5 ± 0.4	2	2	4	1
	hIg	2.4 ± 0.1	3.1 ± 0.3	+0.7 ± 0.2	1	6	1	1
	PBS	2.4 ± 0.1	3.2 ± 0.1	+0.8 ± 0.2	0	7	0	6
C57BL/6	hBCMA-Fc	2.4 ± 0.1	2.1 ± 0.2	−0.3 ± 0.2	4	1	1	1
	hIg	2.2 ± 0.2	2.4 ± 0.2	+0.2 ± 0.1	3	4	0	0
	PBS	2.2 ± 0.2	2.4 ± 0.2	+0.2 ± 0.2	3	4	2	2

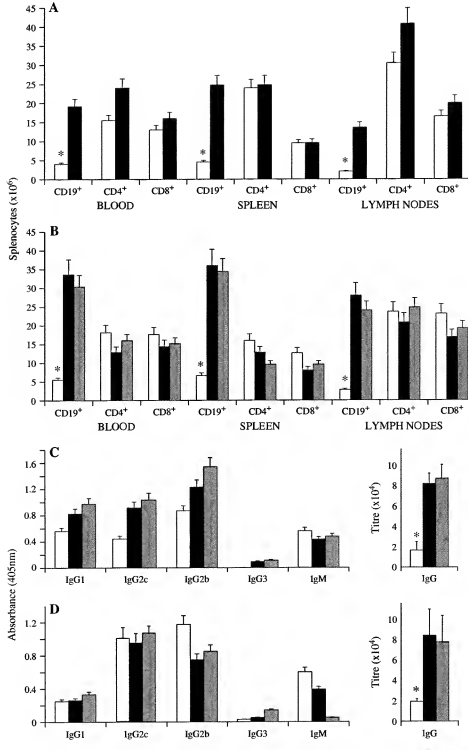

Fig. 5. Treatment of clinically ill mice with hBCMA-Fc blocks B cell survival and reduces rMOG antibody levels. Mice displaying well-established clinical symptoms of EAE treated with hBCMA-Fc (white bars), hIg (black bars) and PBS (grey bars) were sacrificed and analysed by flow cytometry for the percentage of B cells (CD19+), T_h cells (CD4+CD3+) and CTL (CD8+CD3+) in various organs. hBCMA-Fc significantly reduced the number of B cells in all organs examined from (A) NOD/Lt (hBCMA-Fc, $n = 6$; hIg, $n = 8$; $^*P < 0.0001$) and (B) C57BL/6 (hBCMA-Fc, $n = 8$; hIg, $n = 9$ and PBS, $n = 6$; $^*P < 0.0001$). Relative levels of anti-rMOG isotypes and titre of total rMOG-specific IgG ($^*P < 0.05$ for both strains) in serum following treatment in (C) NOD/Lt and (D) C57BL/6 were determined by ELISA. Results represent mean comparable binding of each group ± SEM. The mean titre ± SEM is defined as the sera dilution giving an $OD_{492\ nm}$ three times higher than that of background. Assays were performed on days 54 and 51 post-immunization for NOD/Lt and C57BL/6, respectively.

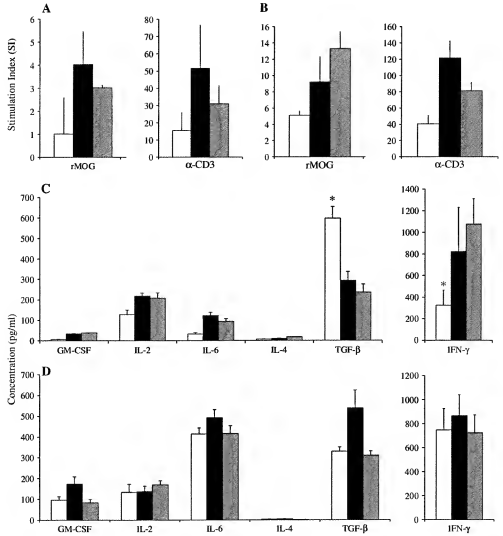

Fig. 6. hBCMA-Fc suppresses T cell activation *in vivo*. Splenocytes from (A) NOD/Lt (hBCMA-Fc, $n = 6$; hIg, $n = 8$ and PBS, $n = 2$) and (B) C57BL/6 (hBCMA-Fc, $n = 8$, hIg, $n = 9$ and PBS, $n = 6$) mice treated with hBCMA-Fc (white bars), hIg (black bars) and PBS (grey bars) were re-stimulated *in vitro* with rMOG and anti-CD3 mAb for 72 h. Bars represent the mean stimulation index [SI; mean counts per minute (c.p.m.) in wells containing rMOG or anti-CD3 mAb divided by the mean c.p.m. in wells containing RPMI only] ± SEM. Mean c.p.m. with media alone were 1157 (hBCMA-Fc), 1918 (hIg), 1385 (PBS) c.p.m. for NOD/Lt and 2136 (hBCMA-Fc), 2045 (hIg), 1967 (PBS) c.p.m. for C57BL/6. Splenocytes from (C) NOD/Lt (IFN-γ: *$P < 0.05$; TGF-β. $P < 0.008$) and (D) C57BL/6 mice were cultured in the presence of rMOG for 48 h, the supernatants were then removed and cytokine concentrations determined by ELISA. Results obtained on days 54 and 51 post-immunization for NOD/Lt and C57BL/6, respectively.

function in initiating the onset of clinical symptoms in this disease. Hence, that hBCMA-Fc pre-treatment reduced the frequency and delayed the onset of MOG-EAE induction may be due to its negative effects on T cell activation as well as the production of antibodies directed against MOG.

It appears unlikely that pre-treatment with BCMA-Fc would prevent or reduce the establishment of MOG-specific germinal centres (GCs) in the SP of immunized mice since both BAFF$^{-/-}$ mice or mice containing a mutated form of the BAFF-R (A/WySnJ) are able to produce a similar frequency of GCs giving rise to somatically mutated B cells (46). Furthermore, BCMA-Fc-treated mice have previously been shown to initiate GC production; however, these fail to persist owing to a failure to develop a mature follicular dendritic cell (FDC) reticulum and thus the failure to capture immune complexes (47), a phenotype very similar to that of the BAFF$^{-/-}$ mice. Thus, the reduction in B cells following BCMA-Fc treatment before or after MOG immunization would not appear to be a limiting factor in the generation of MOG-specific antibody but rather in the maintenance of the FDC reticulum in the GC. Recently, FDCs in human LNs were found to abundantly express BAFF on their surface and thus could act as a local source of BAFF to GC B cells (48). It is possible that the absence of BAFF, or blocking BAFF activity, prevents the recruitment or persistence of FDCs by preventing interactions with BAFF-R on B cells in the GC.

The reduction in the number and extent of plaque formation in hBCMA-Fc-treated mice is likely to reflect the dramatically

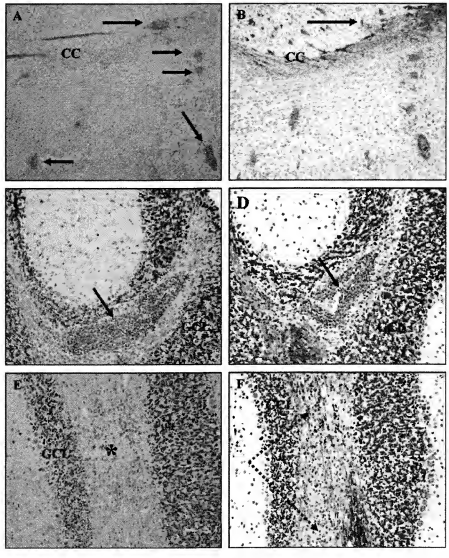

Fig. 7. hBCMA-Fc inhibits CNS inflammation and tissue damage in EAE. Histological analysis of brain sections stained with (A, C, E) H&E and (B, D, F) LFB. NOD/Lt mice treated with (A, B) hlg, mid-brain (C, D) hlg, cerebellum, (E, F) hBCMA-Fc, cerebellum. Arrows with solid lines indicate inflammatory lesions. Region between the arrowheads of dotted lines indicates the area of demyelination. Asterisk in (E) represents older inflammatory lesion. GCL, granule cell layer; CC, corpus callosum. (A & B = ×50, C–F = ×100 magnification.)

impaired humoral response against conformation-dependent epitopes of MOG. Antibody-dependent cell cytotoxicity such as FcR-mediated phagocytosis and activation of NK cells through CD16 would obviously be reduced as a result.

Recently, it has been shown that BAFF is expressed in the CNS during MOG-EAE (49). Although we did not directly measure BAFF expression in the CNS following the various treatments, it is possible that sequestration of BAFF in the brain by hBCMA-Fc would impair the survival of the infiltrating B cells. In addition, reduction of T cell co-stimulation due to a lack of BAFF-mediated signalling may decrease the inflammatory response, thus contributing to the observed histology. Interestingly, the demyelinating plaques present in mice treated with hBCMA-Fc post-disease onset were restricted to the spinal cord and cerebellum and do not appear to have developed recently, as the inflammatory infiltrate is diffuse and sparsely populated. In striking contrast, multiple active lesions were widely dispersed throughout the CNS in control-treated mice. These findings support the notion that hBCMA-Fc treatment is able to restrict the inflammatory process; thus, the demyelinating plaques present would have most likely occurred during the onset and early stages of the disease, before or near the commencement of treatment. Therefore, the timing of treatment would be a vital parameter in the effectiveness of this approach in EAE, with earlier treatment (clinical score = 1) likely to result in less lesion formation, thus a greater degree of recovery.

While NOD/Lt mice administered with hBCMA-Fc displayed far less inflammatory foci and demyelinating plaques than control-treated mice, their tissue damage was also less severe

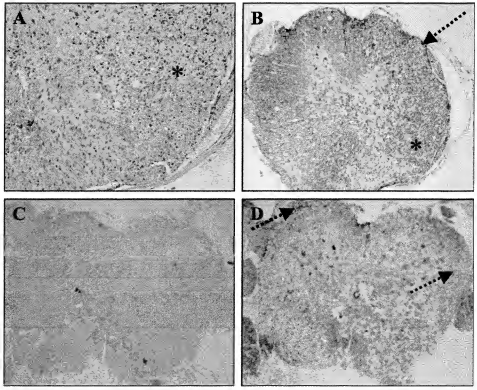

Fig. 8. Histological analysis of NOD/Lt spinal cord in mice treated with (A and B) hIg and (C and D) hBCMA-Fc. (A and C) H&E, (B and D) LFB. Arrowheads of dotted lines indicate demyelination. Asterisk represents area with intense inflammatory lesion. Magnification: A = ×50; B–D = ×20.

than the C57BL/6 mice receiving hBCMA-Fc treatment. This reflects the level of clinical recovery between mouse strains following the onset of hBCMA-Fc treatment. A possible reason why NOD/Lt mice responded more favourably to treatment than C57BL/6 mice is the difference in the predominant subtype of T_h cells and secreted cytokines. While the exact mechanism remains to be fully determined, hBCMA-Fc treatment post-disease onset leads to an up-regulation of TGF-β levels in NOD/Lt mice that were not present in C57BL/6 mice. This is consistent with the large decrease in IFN-γ production in NOD/Lt mice and an unaltered level in C57BL/6 mice. It is also possible that the lower titre of anti-MOG antibody observed in the NOD/Lt (1/13 333) compared with C57BL/6 mice (1/23 000) or the role of MOG-specific antibody in EAE pathogenesis varies between strains and that these factors also affect the responsiveness of each strain to treatment. It should be noted that in the pre-treatment experiment, we did observe a similar increase in the rMOG-stimulated splenocyte secretion of TGF-β and concomitant decrease in secretion of IFN-γ in the hBCMA-Fc-treated C57BL/6 strain. The reduction in the serum anti-MOG antibody IgG2c isotype (39) (T_h1 response) observed in the pre-treatment experiment and in the NOD/Lt mice in the treatment experiment also coincides with the T_h2/T_h3 response of rMOG-stimulated T cells from these mice. The fact that we did not see this reduction in the C57BL/6 mice for the treatment experiment also coincides with the T_h1 phenotype of T cells observed for these mice. Overall, it is possible that the difference in the response to the treatments between strains is a reflection of the therapeutic window in each strain being different.

Our results support the evidence that the actions of BCMA ligands, particularly BAFF, are not restricted to B lymphocytes but, as mentioned earlier, also act as strong co-stimulators of T lymphocyte activation (24, 36–38). Despite having a greater percentage of T cells than control samples (Figs 2A and B and 5A and B), splenocyte cultures from mice treated with hBCMA-Fc had a noticeably weaker proliferative response to rMOG and anti-CD3 (Figs 3A and 6A). Since no hBCMA-Fc was added into the culture, these results suggest that sequestration of BAFF suppresses the activation of T cells *in vivo* and supports other findings that hTACI-Fc treatment reduces the *in vivo* activation of CL-specific T cell proliferation in a mouse model of rheumatoid arthritis (37). Also in accordance with this finding, we observed that hBCMA-Fc inhibits the antigen-specific production of pro-inflammatory cytokines in mice responding positively to the treatment. The significant increase in the production of the anti-inflammatory cytokine TGF-β in the hBCMA-Fc-treated NOD/Lt mice is quite surprising and has not been reported previously using BAFF/APRIL decoy receptors. The mechanism behind this altered cytokine profile is not clear. It is possible that BAFF not only co-stimulates T cells but also delivers a pro-inflammatory polarizing signal. Although if this were the case, one might imagine a more severe phenotype in BAFF$^{-/-}$(34, 35) mice given the important role of pro-inflammatory cytokines in development. Our findings indicate that the suppressive

nature of hBCMA-Fc treatment on T lymphocytes does not appear to be restricted by antigen specificity (since reduced proliferation to anti-CD3 is observed), and therefore is most likely to function by directly blocking BAFF interacting with its cognate receptor on T cells.

These investigations firmly support a role for BAFF and/or APRIL in the demyelinating autoimmune model, MOG-EAE. As a direct result of targeting BCMA ligands, we were able to not only inhibit the survival of B cells and Ig production but also modify T cell activity and cytokine production. A greater knowledge of the effect of BAFF on other cell types, besides B cells, coupled with an understanding of APRIL signalling should give greater insight into the therapeutic mechanisms of hBCMA-Fc in EAE. As it stands, our findings indicate that BAFF- and/or APRIL-mediated signalling are important in the pathogenesis of MOG-EAE, and that blocking these signals may prove an effective strategy of immunomodulation for the human disease of MS.

Previous attempts to induce peripheral T cell tolerance to MOG, via immune deviation therapy, have been unsuccessful due to the development of pathogenic MOG antibody-mediated demyelination (6, 50). While the precise immune mechanisms underlying the therapeutic role of hBCMA-Fc on MOG-EAE remain to be determined, our findings indicate that both the cell-mediated and humoral immune responses are either directly or indirectly influenced. These beneficial effects highlight the importance of targeting both responses for therapeutic intervention in demyelinating diseases such as MS.

Acknowledgements

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Abbreviations

APC	antigen-presenting cells
APRIL	a proliferation-inducing ligand
BAFF	B cell-activating factor of the tumour necrosis factor family
BCMA	B cell maturation antigen
CL	collagen
CNS	central nervous system
DLN	draining lymph node
EAE	experimental autoimmune encephalomyelitis
FDC	follicular dentritic cell
GC	germinal center
GM-CSF	granulocyte macrophage colony-stimulating factor
H&E	hematoxylin and eosin
LFB	Luxol fast blue
LN	lymph node
MAG	myelin-associated glycoprotein
MOG	myelin oligodendrocyte glycoprotein
MOG-EAE	MOG-induced EAE
MS	multiple sclerosis
NOD	non-obese diabetic
PB	peripheral blood
PLP	proteolipid protein
rMOG	recombinant myelin oligodendrocyte glycoprotein
SLE	systemic lupus erythematosus
SP	spleen
TACI	transmembrane activator and CAML-interactor
TGF	transforming growth factor
TNF	tumour necrosis factor
[^{3}H]TdR	[^{3}H]thymidine

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